AD_____

GRANT NUMBER DAMD17-97-1-7069

TITLE: Differential Regulation of Cell Cycle Progression in Human Breast Cancer Cell Lines by the Estrogen Receptor

PRINCIPAL INVESTIGATOR: James Direnzo, Ph.D.
Dr. Myles Brown

CONTRACTING ORGANIZATION: Dana Farber Cancer Institute
Boston, Massachusetts 02115

REPORT DATE: August 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1999	Annual (1 Aug 98 - 31 Jul 99)

4. TITLE AND SUBTITLE

Differential Regulation of Cell Cycle Progression in Human Breast Cancer Cell Lines by the Estrogen Receptor

5. FUNDING NUMBERS

DAMD17-97-1-7069

6. AUTHOR(S)

Direnzo, James, Ph.D.
Dr. Myles Brown

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dana Farber Cancer Institute
Boston, Massachusetts 02115

E*Mail: james_direnzo@dfci.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT

Critical predictions as to the biological behavior, and thus the appropriate therapeutic strategy, of breast cancers can be made based upon the status of the estrogen receptor (ER). In support of DOD grant # DAMD17-97-1-7069, our goal is to better understand the mechanisms by which ER controls the expression of target genes and therefore mediates the biological effects upon gene regulation and cell cycle progression. Our detailed studies of the regions of ER that control cell cycle progression in breast cancer cell lines have indicated an absolute requirement for the Activating Function-2 (AF-2) region of ER for hormone-dependent cell cycle progression. In many different classes of nuclear receptors, this area has been vigorously studied and has been shown to be important for the physical interaction between hormone bound receptors and coactivators. Our studies have demonstrated that mechanisms involving the chemical and structural modification of chromatin are critical for transcriptional responses to estrogen and may also be important for estrogen-dependent cell cycle progression.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health. JDR

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules. JDR

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories. JDR

J. D. Ray 9/23/99

PI - Signature Date

Table of Contents

Introduction

Breast cancer is the most common cancer afflicting women in the United States and the second leading cause of cancer death. A detailed understanding of the molecular mechanisms involved in the initiation and progression of breast cancer will likely lead to better methods for prevention, detection and treatment. Critical predictions as to the biological behavior, and thus the appropriate therapeutic strategy, of breast cancers can be made based upon the status of the estrogen receptor (ER). Approximately 50% of all breast cancers are characterized by elevated levels of ER and, of these, approximately 2/3exhibit estrogen-dependent growth. In addition, the loss of ER expression in advanced breast cancers, is a hallmark of the transition from a cancer that is hormone dependent, to one that is refractory to antiestrogen treatment, and has significantly greater metastatic tendencies. Such cancers are significantly more difficult to treat and are correlated with poor patient survival. A greater understanding of the effects of ER upon cell cycle regulation in breast cancer will likely lead to the identification of new potential targets of pharmacological intervention against breast cancer.

Mapping the Regions of ER Responsible for Cell Cycle Progression

During the past year, our approach to understanding the role of ER in the initiation and progression of breast cancer has been to express recombinant forms of ER, in breast cancer cell lines. Overexpression of wild type and mutant forms of ER in the ER-positive and estrogen-dependent MCF-7 cells have provided strong evidence that the C-terminal Activating Function-2 (AF-2) is required for estrogen-dependent cell cycle progression. This conclusion is based upon two different types of experiments. In the first, stable MCF-7 derived cell lines were developed that overexpressed four different versions of ER. These forms were wild-type ER (1-596) ER-Δ AF-1 (174-596), ER–Δ AF-2 (1-534) and ER Δ AF-1/2 (174-534). All versions of ER were cloned into an expression vector that contained a selectable marker for neomycin resistance. These plasmids were transfected into MCF-7 cells and neomycin resistant colonies were cultured and subcloned. Expression of the various forms of the receptor was confirmed by western blot analysis. In these studies, it was observed that neomycin resistant colonies expressing wild type ER and ER-Δ AF-1 grew robustly and retained their hormone-dependence. In contrast neomycin resistant colonies expressing ER–Δ AF-2 and ER Δ AF-1/2 were fewer in number and grew very slowly. This growth inhibition was not rescued by estrogen treatment. The observation that these colonies were much less abundant than those expressing wild-type ER or ER-Δ AF-1, suggests that the cells expressing forms of ER that lacked the AF-2 were at a significant growth disadvantage. The small number of colonies that did grow out of these experiments were neomycin resistant and did proceed through the cell cycle in a manner that was resistant to the growth inhibitory effects of anti-estrogens. Taken together, these studies strongly demonstrated that the AF-2 of ER was required for estrogen-mediated cell cycle progression. This result is consistent with previous studies that suggested that the ability of ER to activate transcription of a target gene in response to hormone is also dependent upon the AF-2.

In order follow up on these studies and to try to gain a better understanding of the function of the AF-2 domain of ER the same four versions of ER were cloned into a replication-defective retroviral expression vector. Transfection of this vector into a amphotrophic retroviral packaging cell line, called Bing cells, produced replication defective retroviruses that could direct the expression of the ER versions described above and were selectable with puromycin. These recombinant viruses were used to infect wild-type MCF-7 cells. In these studies it was observed that the ER lacking the AF-2 exerted a dominant negative effect over the endogenous ER. This effect was seen in dramatically reduced transcriptional responses to estrogen and to an effective block of cell cycle progression at the G1/S boundary. Similar retroviral vectors were made that allowed antibiotic selection with phleomycin, making them useful for additional experiments in cells that were previously made to be resistant to puromycin. These experiments will be described later in this manuscript.

Establishment of Immortalized Mammary Epithelial Cells (IMECs)

Virtually all breast cancers arise from mammary epithelial cells. These cells are the primary structural elements of the milk-producing ducts of the mammary gland. Efforts to study these primary mammary epithelial cells have been hampered by the fact that when grown in culture, they undergo growth arrest and become senescent. Studies in several laboratories have shown that cellular senescence is under the control of the enzyme telomerase. Telomeres are essential elements found at the ends of chromosomes. These long repetitive DNA sequences protect the chromosomes from degradation, end-to-end fusion and libation. It has now been conclusively shown that with each round of cell division results in the shortening of the telomeres. In this way, the telomeres serve as a sort of biological clock that counts the number of replications a cell has undergone. Eventually telomeres become so short that the chromosomes become damaged and the cells undergo senescence. The identification and cloning of the catalytic subunit of the telomerase complex has made it possible to prolong the life a primary cell in culture by maintaining telomeric length.

Primary mammary epithelial cells (MECs) can be obtained commercially from a company called Clonetics. These cells will grow for several passages in a chemically defined growth medium before becoming senescent. During this short period of growth in culture, these cells are susceptible to infection by recombinant retrovirus. Our attempt to prolong the life of the MECs would be via overexpression of telomerase from a retroviral vector. This vector, pBABE-puro hTERT, and the empty vector control, pBABE-puro were transfected into a viral packaging cell line and the recovered viruses were added to the MECs in culture. Following viral infection, cells were selected in puromycin. Both viruses produced puromycin resistant colonies. These colonies were grown in pools and cultured through several passages. After 6 passages, the MECs that had been infected with the empty vector control began to show the visual signs of senescence, while those infected with the telomerase expression virus appeared to grow continually. These cells are now at passage 50 and appear to be completely resistant to senescence, suggesting that they have been immortalized. It is important to note that telomerase is not an oncogene and that these Immortalized Mammary Epithelial Cells (IMECs) are not transformed. These efforts have established a mammary epithelial cell line that can be carried indefinitely in culture and will be useful for several studies aimed at understanding the minimum requirements for oncogenic transformation, the role of ER in both healthy and cancerous mammary cells. Since they are not transformed they are much more likely to maintain their genomic integrity which may make them useful for homologous recombination-mediated gene targeting, or gene knockouts.

A very interesting and potentially informative observation was made while culturing and analyzing the IMECs. What was observed was that if these cells were fed every second day and passage at approximately 90% confluence they would continue to

proliferate. However if they were taken off of the two-day feeding schedule, they would undergo differentiation. In this differentiation, it was observed that the cells would begin to arrange themselves into structures that resembled mammary ducts. Long lines of cells that appear to be the result of multiple rounds of symmetric replication would form and would ultimately enclose areas that resemble luminal spaces in a mammary duct. Currently studies using immunoflourescence are being done to determine if these cells have acquired the biochemical markers of luminal epithelia. If this proves to be the case, it will represent the first in vitro system for the study of the development and differentiation of human mammary ducts. Since these cells are grown in a chemically defined growth media, which is supplemented with Epidermal Growth Factor (EGF) insulin, prolactin and hydrocortisone, experiments were done to ask which if any of these factors was required for the observed differentiation. Preparations of cell culture media that lacked each of the mentioned factors were used to culture the IMECs. What was observed in these studies was that while both EGF and insulin were required for the cells to continue to grow, their absence did not prevent the differentiation. Cell cultured in the absence of insulin grew slowly for 3-4 days by which time they did appear to differentiate. Cells grown without EGF grew somewhat longer and differentiated, but ultimately died as well. Surprisingly, cells grown in the absence of prolactin were unaffected. Their growth and differentiation was very similar to IMECs grown in complete media. Surprisingly, IMECs grown in the absence of hydrocortisone grew normally, but failed to differentiate. These experiments were carried out over the course of 10 days. Even at the end of this time, the IMECs grown without hydrocortisone were observed to be progressing through the cell cycle and were not forming any of the multicellular structures that were plainly apparent in the control IMECs which were cultured in completed medium. These studies suggested that another member of the nuclear receptor superfamily; the glucocorticoid receptor (GR) is required for the observed differentiation of the IMECs. Further studies regarding this interesting phenomenon are now in the planning stage.

Our primary goal in using the IMECs was to determine if they were responsive to estrogen, and which of the known proteins that mediate estrogen responsiveness were expressed. Using both western blot analysis and the ultra-sensitive RT-PCR, it was determined that these cells do not express an endogenous ER α or ER β. Similar analyses, however did determine that the three members of the Steroid Receptor Coactivator (SRC) family were all expressed as well as another known coactivator of estrogen signaling the Brahma Related Gene-1 (BRG-1). These observations suggested that, as a "null cell line" the IMECs were likely to be useful in the study of ER function in mammary epithelia. Since these cells were already made to be puromycin resistant, the four previously described versions of ER were cloned into retroviral vector that would confer resistance to phleomycin. Using a similar packaging and infection strategy, phleomycin resistant IMECs were derived that expressed wild-type ER and the AF-1 and AF-2 mutants described above. These cells appear to be very similar to the parental IMECs in that they grow and differentiate in a manner that is unaffected by the presence of the various forms

of ER. Western blot analysis has confirmed the expression of wild-type ER and the ER lacking the AF-2 domain. Transient transfection of these cell lines with an estrogen-responsive reporter gene (ERE-tk-luciferase) showed that expression of wild type ER and the ER lacking the N-terminal AF-1 was sufficient to confer a transcriptional response to estrogen, while the ER lacking the AF-2 appeared to suppress transcription in response to estrogen. It is currently unclear as to why the ER Δ AF-1 was not detected in western blot analysis, but appear to confer an estrogen response, but it is believed to be due to technical difficulties. The observation that these cells were responsive to estrogen in a transfection suggests that the ER Δ AF-1 is being expressed. It is also interesting to note that in these studies, the partial estrogen agents 4 hydroxy-tamoxifen acted as a potent antiestrogen, consistent with its biological effects in the breast. Detailed studies of the effects of ER and the associated mutants on the differentiation of IMECs are ongoing.

Biochemical Analysis of the Coactivation of Estrogen Signaling

The relative ease of activation of the nuclear receptors has made them a very useful system for understanding the molecular mechanisms by which a hormone binding signal is transmitted to basal transcription machinery. These studies began with the search for putative coactivators of receptor signaling. One family of factors the SRC-1 family was shown to be important for receptor signaling in that they interacted with nuclear receptors in manner that is both hormone and AF-2 dependent. Studies of the effects of SRC-1 on ER activity were enhanced by the development of monoclonal antibodies directed against SRC-1. These antibodies were useful in definitively showing that SRC-1 did indeed interact with ER in response to estrogen. One of these antibodies was shown to interact with BRG-1. BRG-1 is an important member of the chromatin remodeling Swi/Snf complex, and had been shown to be an important regulator of nuclear receptor activation. With the exception of a small region of the C-terminus of SRC-1, BRG-1 is structurally unrelated to SRC-1. It is likely that this region of similarity accounts for the cross-reactivity observed between the anti-SRC-1 antibody and BRG-1. The observation that BRG-1 cross-reacted with one of the anti-SRC-1 antibodies suggested that the previously reported Coactivation of nuclear receptor signaling by BRG-1 may be through mechanisms similar to those of SRC-1.

Our studies of BRG-1 have shown that, like SRC-1 BRG-1 associates with ER in a manner that is hormone and AF-2 dependent. Furthermore the use of an adrenal carcinoma cell line, SW-13, which is devoid of BRG-1 and its close relative Brahma has made it possible to show that BRG-1 is strictly required for ER signaling. Transient transfection studies using these cells showed that ER was incapable of transcriptional activation in the activation of BRG-1. Interestingly, two other important coactivators of ER, SRC-1 and the CREB-Binding Protein (CBP) were incapable of coactivating ER signaling in the absence of BRG-1. These studies suggested that BRG-1 and its chromatin remodeling activity were critical factors that influence the action of the ER. The

observation that overexpression of CBP could enhance BRG-1 mediated transcriptional activity, coupled to the reports that CBP contained a potent histone acetyltransferase (HAT) activity, suggested that the ability of BRG-1 to coactivate ER signaling may be enhanced by an increased acetylation state within the cell. To test this hypothesis, the potent inhibitor of histone deacetylation, Trichostatin A (TSA) was tested for its ability to enhance BRG-1 mediated coactivation of ER signaling. In these studies, it was observed that TSA dramatically enhanced the ability of BRG-1 to coactivate ER signaling. It is believed that this synergy may be mediated by a region of BRG-1 called the Bromodomain. The bromodomain is a region of BRG-1 that is conserved in a large number of nuclear proteins that are implicated in the chemical and structural modification of chromatin. Recent studies of the p300/CBP Associated Factor (PCAF) revealed that the bromodomain consists of four helices arranged in a left handed bundle that forms the binding pocket for acetyl-lysine. This finding has lead to the hypothesis that the synergy observed between TSA and BRG-1 may be mediated by an interaction between the bromodomain of BRG-1 and acetylated nucleosomes. Mutagenesis studies of the bromodomain of BRG-1 are ongoing to test this hypothesis.

Key Research Accomplishments

James DiRenzo Ph.D.

Award # DAMD17-97-1-7069

- Identification of the AF-2 region of ER as the region critical for hormone-dependent cell cycle progression.

- Biochemical and functional characterization of the ER coactivators, SRC-1, BRG-1 and CBP.

- Demonstration that BRG-1 is strictly required for proper ER function and that both SRC-1 and CBP require BRG-1 in order to exert their coactivational effects on ER signaling.

- Overexpression of human telomerase in primary mammary epithelial cells leading to putative immortalization .

- Overexpression of wild-type and mutant forms of ER in the putatively immortalized mammary epithelial cells.

- Ongoing development of an in culture mammary epithelial cell differentiation system and determination of the critical hormones involved in the differentiation.